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THREE-YEAR SURVEY OF THE EPIDEMIOLOGY OF ROTAVIRUS, ENTERIC ADENOVIRUS, AND SOME SMALL SPHERICAL VIRUSES INCLUDING "OSAKA-AGENT" ASSOCIATED WITH INFANTILE DIARRHEA

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SUMMARY Studies were made by electron microscopy (EM) on the viruses associated with diarrhea of outpatients at a pediatric clinic in Osaka Prefecture during the three year period from 1980 through 1982. The viruses detected by EM by negative staining with phosphotungstic acid (PTA) were classified morphologically into 6 groups: rotavirus, adenovirus and four kinds of small spherical viruses, calicivirus, astrovirus, picornavirus / parvovirus (P/P)-like agent and Osaka-agent. Osaka-agent seems to be a newly identified small virus. It is 35–40 nm in diameter with a fringe of spike-like structures on its surface. Viruses were detected in 181 of the 395 cases of diarrhea (45.8%). Rotavirus was detected in 122 (30.9%) of the total cases and in 67.4% of the virus-positive cases, while other viruses were detected in 15% of the total cases; adenovirus in 23 (6%) and small agents in 36 (9%). Rotavirus infection showed a distinctive seasonal variation, being mainly restricted to cooler months, but infections with other viruses did not show any seasonal variation. The age distribution of patients suggested that infants of 0 to 2 years old are very susceptible to all viruses. Attempts to cultivate these viruses in vitro were successful with only two isolates of adenovirus type 5.

INTRODUCTION

Rotavirus is a major pathogen associated with acute gastroenteritis of infants and young children. Norwalk virus, adenovirus, coronavirus and some small spherical virus particles including calicivirus and astrovirus are also important etiological agents closely related with diarrhea, as reported in recent reviews (Holmes, 1979; WHO, 1980; Murphy, 1981; Barnett, 1983; Cukor and Blacklow, 1984). These viruses have been found in many parts of the world and seem to have unique geographic distributions that may be influenced by the weather and climate of the areas examined (Middleton et al., 1977; Hieber et al., 1978; Brandt et al., 1979; Murphy, 1981; Konno et al., 1983). Therefore, the viruses detected and their seasonal variations probably differ in different areas. Some of these viruses have caused unexpected mass outbreaks of acute gastroenteritis in susceptible populations. Indeed there were some outbreaks of acute gastroenteritis associated with rotavirus or calicivirus among school-age children and infants even in Osaka (Oishi et al., 1979; Oishi et al., 1980). Thus it is necessary to study the epidemiological status of the etiological agents of acute gastroenteritis for prevention of these viral infectious diseases.

We examined the etiological agents associated with diarrhea of outpatients at a pediatric clinic in Toyonaka City, Osaka Prefecture, by electron microscopy over a three year period. This paper reports the results of this study and describes a new small spherical virus particle with a fringe of spike-like structures that was named " Osaka-agent ".

MATERIALS AND METHODS

1. *Patients and specimens*

In all, 395 stool specimens from infants and young children of 0 to ten years old with acute diarrhea were obtained from a pediatric clinic. This clinic is located in Toyonaka City in the northern part of Osaka Prefecture. Stool specimens were obtained from patients 1 or 2 days after the onset of diarrhea. The study was from January 1980 to December 1982.

2. *Electron microscopy (EM)*

Stool specimens were prepared as 10–20% suspensions in phosphate buffered saline (PBS) and extracted with an equal volume of fluorocarbon (Daiflon S3, Daikin, Japan). After low-speed centrifugation of the mixture, the supernatant was centrifuged at 100,000 g for 90 min in a Beckman LH ultracentrifuge. The resulting supernatant was removed and the pellet was resuspended in a few drops of PBS. One drop of this suspension was placed on a Formval-carbon-coated grid, and negatively stained with 3% phosphotungstic acid (PTA), pH 6.5. The grid was dried and examined in a Hitachi HU-11 electron microscope.

3. *Virus cultivation*

Attempts to cultivate the viruses were made only on specimens in which virus was identified. These specimens were extracted with fluorocarbon. Then with pretreatment with an equal volume of 10 μg/ml of suspension of cristalline trypsin (Sigma Comp., USA) at 37 C for 30 min, they were inoculated as described by Sato et al. (1981) into duplicate culture tubes containing monolayers of MA-104, HEp-2, FL or GMK cells. The cell cultures were incubated at 37 C on roller drums and observed for five to six days for cytopathic effects (CPE) under a microscopy. Some specimens were also inoculated intraperitoneally into one-day-old mice.

4. *Meteorological data*

Data on monthly air temperature and relative humidity in Osaka Prefecture were obtained from annual and monthly reports of the Japan Meteorological Agency. Mean monthly temperatures and relative humidities in the three years were calculated from these data.

RESULTS

1. *EM examination*

Viruses detected by EM by PTA-negative staining were classified morphologically into 6 groups: rotavirus, adenovirus, and four kinds of small spherical type viruses. The small particles were calicivirus and astrovirus, with the characteristic configurations by negative staining described by Madeley (1979), picornavirus / parvovirus-like particles (P/P)

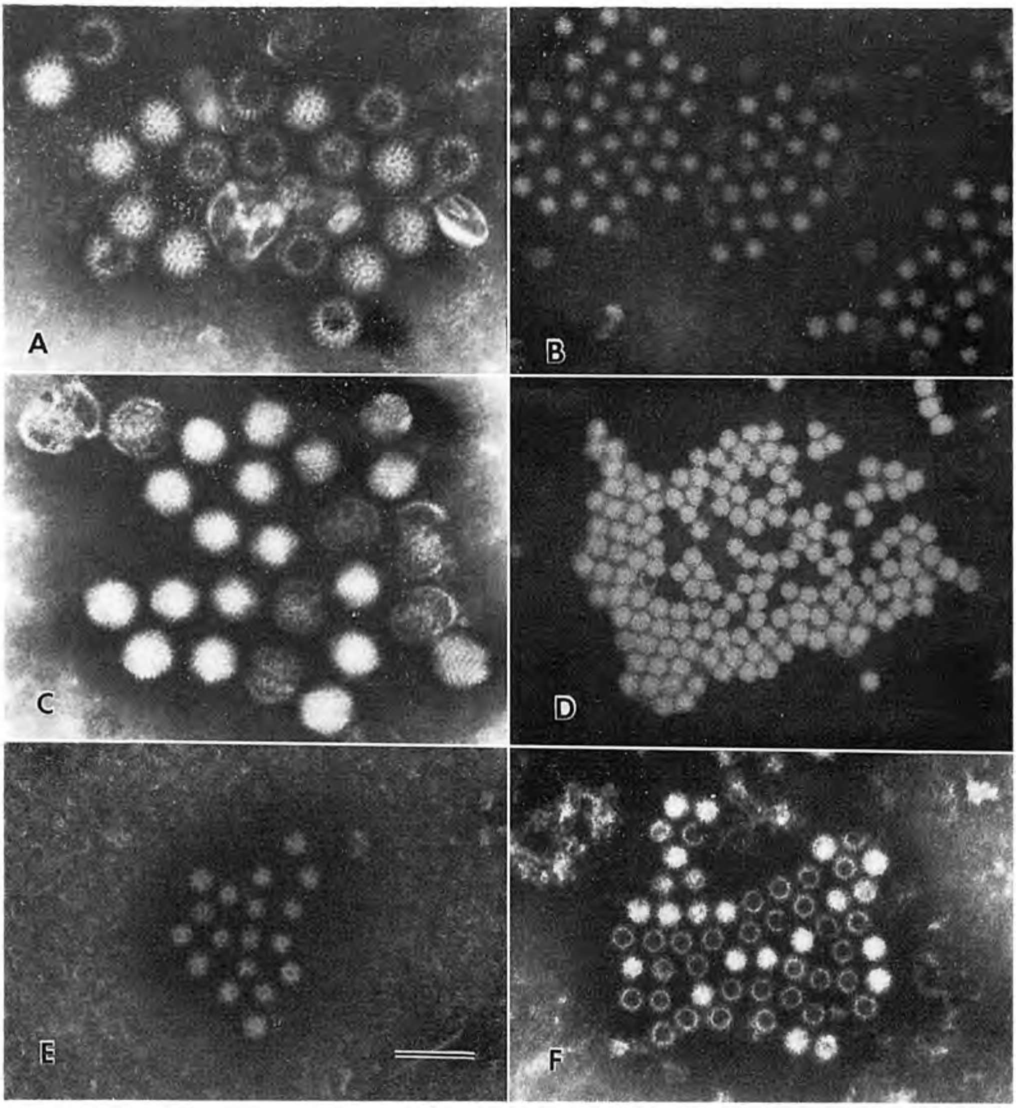

FIGURE 1. Viruses detected in stools of infants with diarrhea. A, Rotavirus; B, Astrovirus; C, Adenovirus; D, Picornavirus/parvovirus-like particles; E, Calicivirus; F, Osaka-agent. Scale: 100 nm. ×108,000.

of 25–30 nm diameter with a smooth surface, and particles of 35–40 nm diameter (average 40 nm) with a fringe of spike-like structures. Fig. 1 shows the appearance on PTA-negative staining and the size of these virus particles. The particles of 35–40 nm diameter (Fig. 1-F) were named " Osaka-agent ". Rotavirus, adenovirus, astrovirus and Osaka-agent were often observed as big clumps of particles in stool suspensions, as illustrated in Fig. 2. In addition, Osaka-agent was seen as empty particles as well as complete ones, as shown in

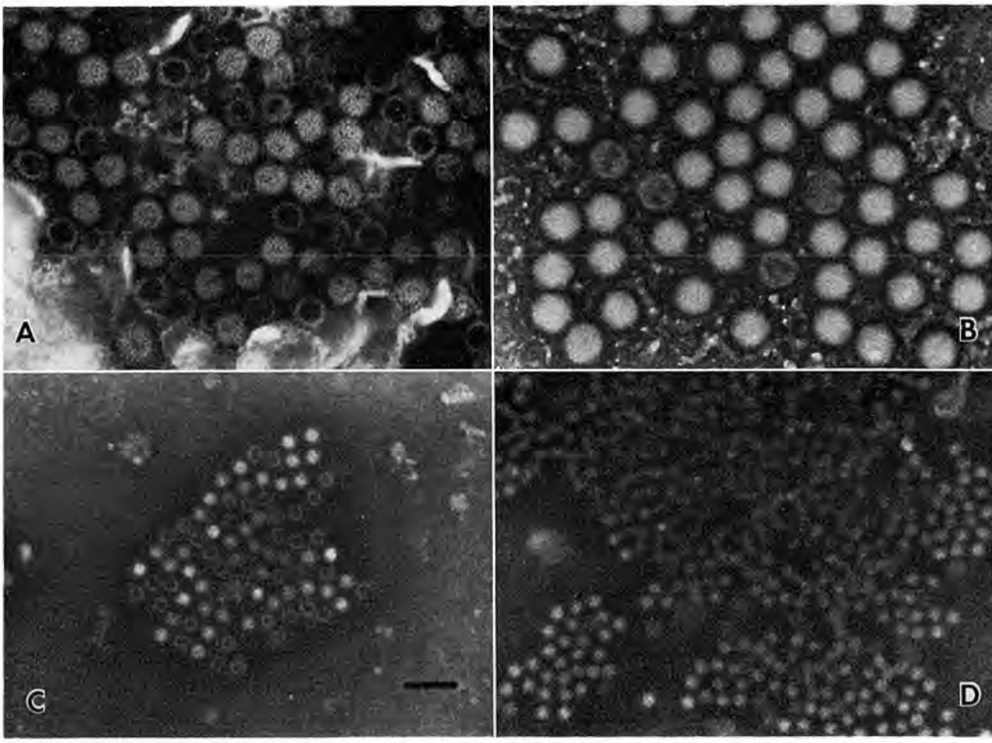

FIGURE 2. Characteristic clumps of rotavirus, adenovirus, astrovirus and Osaka-agent detected by EM in stool specimens. A, Rotavirus; B, Adenovirus; C, Osaka-agent; D, Astrovirus. Scale: 100 nm. ×72,000.

Fig. 1-F and 2-C. These small spherical agents were easy to identify by direct EM. Mixed infections with rotavirus and adenovirus, and rotavirus and P/P, respectively, were seen in 2 of 395 specimens.

2. *Yearly incidences of viruses*

Table 1 shows the yearly incidences of patients and virus-positive cases. Viruses were found in 181 of 395 cases (incidence, 41–50%; mean 45.8%). Rotavirus was detected by EM in 122 (30.9%) of 395 cases and in 67.4% of 181 cases with virus. Others were detected at lower incidences: adenovirus in 23 cases (5.8% of the total cases); astrovirus in 9 cases (2.3%); calicivirus in 4 cases (1.0%); Osaka-agent in 16 cases (4%) and P/P in 7 cases (1.8%) among 395 cases. Cases of Osaka-agent and astrovirus increased slightly during this three-year study period.

3. *Seasonal incidences of viral cases*

The seasonal distributions of cases of viral infections including results on rotavirus from January to March, 1983, are shown in Fig. 3. Cases of diarrhea in infants and young children in this district were more in cooler months, from November to March, and less in warmer monthes each year. Most cases of viruses were detected between December and March, probably because of the high prevalency of rotavirus infections during this period, since cases of infections with other viruses were randomly distributed throughout the year.

TABLE 1. *Yearly incidences of virus-positive cases of infantile diarrhea detected by EM*

Virus	1980	1981	1982	Total	Percentage incidence	
					in virus-positive cases	in total cases
Rotavirus	41	49	32	122	67.4	30.9
Adenovirus	6	6	11	23	12.7	5.8
Calicivirus	2	1	1	4	2.2	1.0
Astrovirus	1	2	6	9	5.0	2.3
Osaka-agent	1	6	9	16	8.8	4.0
Picornavirus/parvovirus-like agent	4	1	2	7	3.9	1.8
Total	55	65	61	181	—	—
	134[a]	130	131	395		
Virus-positive cases (%)	41	50	46.6	45.8	—	—

[a] Total no. of cases examined.

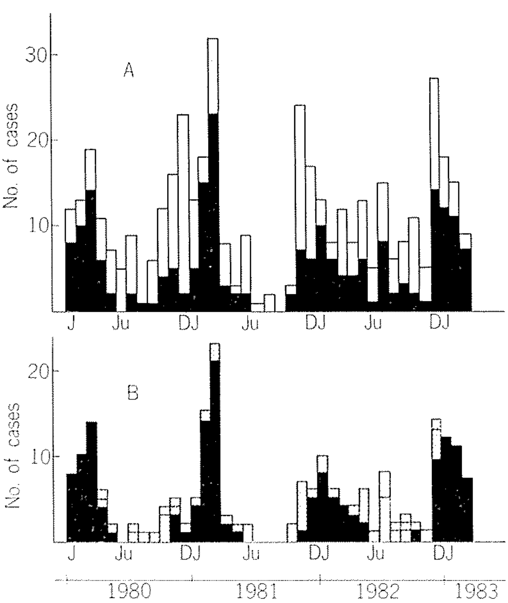

FIGURE 3. Annual distributions of viruses detected by EM in cases of infantile diarrhea. A, No. of cases examined (□) and virus-positive cases (■); B, No. of cases of infection with each virus. ■: Rotavirus, ⊞: Adenovirus, ▨: small spherical viruses.

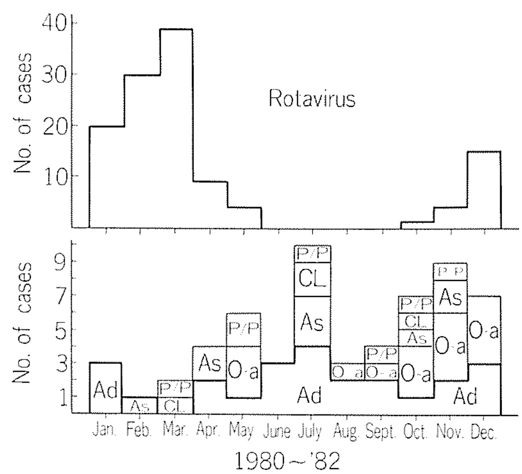

FIGURE 4. Monthly incidences of virus-positive cases of infantile diarrhea. Ad, Adenovirus; Cl, Calicivirus; As, Astrovirus; O-a, Osaka-agent; P/P, Picornavirus/parvovirus-like particles.

4. *Seasonal distributions of viruses detected*

The seasonal distributions of cases of infection with rotavirus and other viruses are shown in Fig. 4. Rotaviruses were found mainly in the cooler months between October and March every year with a peak from December to March. Other viruses were mainly detected in months when rotavirus was no prevalent, although their seasonal variations were not marked: adenovirus and P/P were found in all seasons, calicivirus and astrovirus

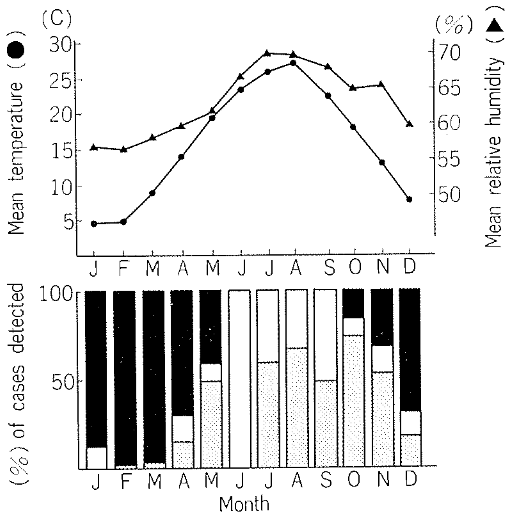

FIGURE 5. Relation of virus-positive cases of infantile diarrhea and weather in Osaka. Mean temperatures and relative humidities in Osaka Prefecture between 1980 and 1982. Incidence (%) was calculated as the No. of cases of infection with a virus/No. of virus-positive cases ×100. ■: Rotavirus, □: Adenovirus, ▤: Small spherical viruses.

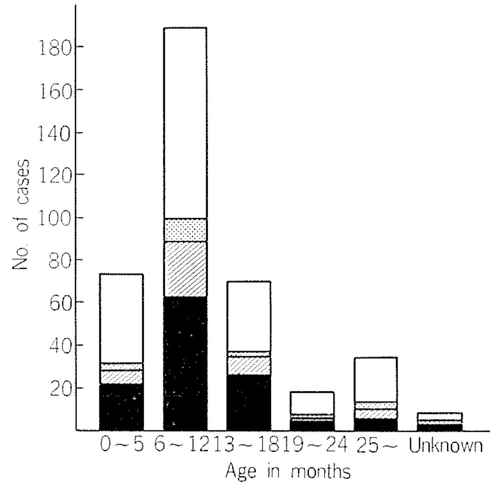

FIGURE 6. Age distribution of cases of infantile diarrhea associated with virus. □: Total No. examined, ■: Rotavirus, ▤: Adenovirus, ▨: Small spherical viruses.

in the summer, and Osaka-agent mainly in the slightly cooler months of October to December.

5. *Weather and virus infection*

Fig. 5 shows the correlations between the monthly mean temperatures and relative humidities in Osaka and monthly incidences of virus infections associated with diarrhea in the three-year period. The incidences of viruses other than rotavirus increased with

TABLE 2. *Age (in month) distribution of virus-positive cases of infantile diarrhea (1980–1982)*

Virus	Median age (months)	Age (in month) 0–5	6–12	13–18	19–24	25–	Unknown	Total
Rotavirus	11	22	62	26	4	5	3	122
Adenovirus	12	3	11	3	1	3	2	23
Calicivirus	14	2	1	0	0	1	0	4
Astrovirus	18	2	4	1	0	2	0	9
Osaka-agent	16	1	7	5	1	2	0	16
Picornavirus/Parvovirus-like agent	10	1	4	2	0	0	0	7
Total	—	31	18	37	6	13	5	181
		74[a]	189	70	19	35	8	395
Virus-positive cases (%)		41.9	47.1	52.9	31.6	37.1	62.5	45.8

[a] Total no. examined

TABLE 3. *Prevalency of virus-positive cases of infantile diarrhea by sex*

Sex	No. examined	Virus-positive	Rotavirus	Adenovirus	Calicivirus	Astrovirus	Osaka-agent	P/P
Male	243	106	74	12	3	4	9	4
		(43.6%)[a]	(30.5%)	(4.9%)		20 (8.2%)		
Female	148	81	56	9	1	5	7	3
		(54.7%)	(37.8%)	(6.1%)		16 (10.8%)		
Total	391	187	130	21	4	9	16	7
		(47.8%)	(33.2%)	(5.4%)		36 (9.2%)		

[a] Numbers in parentheses are percentages.

TABLE 4. *Cases infected with two different viruses within a short period*

Case No.	Sex	Virus	Age of infection (months)	Date	Interval before 2nd infection	Virus	Age of infection (months)	Date
1. Y. S.	M[a]	Adenovirus	9	8-11-'80	3m[b]	Rotavirus	12	11-13-'80
2. A. A.	M	Rotavirus	11	3-4-'81	8m	Astrovirus	19	11-16-'81
3. H. Y.	M	Osaka-agent	13	11-2-'81	16d[c]	Rotavirus	13	11-18-'81
4. Y. Y.	F[d]	Calicivirus	4	1-27-'81	9m	Osaka-agent	12	11-13-'81
5. K. I.	M	Rotavirus	4	1-27-'82	10m	Osaka-agent	14	12-21-'82
6. H. O.	M	Rotavirus	4	3-10-'82	7m	Adenovirus	11	11-26-'82
7. C. T.	F	Adenovirus	5	9-1-'82	6m	Rotavirus	11	3-25-'83
8. C. H.	F	Rotavirus	6yr[e]	3-27-'82	3m	Astrovirus	6yr	7-2-'82

[a] M: male
[b] m: months
[c] d: days
[d] F: female
[e] yr: years

increase in temperature and decreased with decrease in temperature. In Osaka mid-June to mid-July is the wettest and has the highest humidity. There were no cases of rotavirus infection from this time until October. In all three years, no cases of rotavirus infection were found when the mean temperature rose above 23 C and the relative humidity above 63%.

6. *Age distribution*

The age distribution of the incidence is shown in Table 2 and Fig. 6. A very high incidence was observed in children of less than 18 months old [333 (84%) of 395 cases] and various viruses were found in this age group (42 to 53% of the cases). The median age of cases of viral infection was calculated as 10–18 montbs.

7. *Prevalency of virus infection by sex*

Diarrhea was more frequent in boys than girls, although the incidence of virus-positive cases was rather higher in girls, [81 of 148 cases (54.7%) in girls, 106 of 243 cases (43.6%) in boys (Table 3)].

8. *Cases infected with two different viruses within a short period*

During a long-term survey of diarrhea in this district, some infants were noticed to suffer from diarrhea several times within short period. Table 4 summarizes data on cases infected with two different types of virus within a period of months. The infections were with various combinations of viruses, such as rotavirus and adenovirus (Case nos. 1, 6 and 7), rotavirus and astrovirus (Case nos. 2 and 8) and rotavirus and Osaka-agent (Case nos. 3 and 5). In seven of eight cases, one of the pair of viruses was rotavirus, but in Case no. 4 the combination was calicivirus and Osaka-agent. Case no. 3 was infected with rotavirus within one month after Osaka-agent. No case of reinfection with rotavirus was observed.

9. *Virus cultivation*

Attempts to cultivate the viruses in various cultured cells or in suckling mice with treatment of the stool extracts with trypsin were usually unsuccessful, but two strains of adenovirus type 5 (Ad-5) were cultured in vitro.

DISCUSSION

In examinations of the stools of infants and young children with diarrhea at a pediatric clinic in a three year period from 1980 to 1982, we detected six morphologically distinctive virus particles by EM. These were rotavirus, adenovirus and four kinds of small spherical viruses, calicivirus, astrovirus, P/P-like virus particles and Osaka-agent. These virus particles were easily identified by their fine structure when negatively stained with PTA of directly observed by EM. The viruses were closely associated with diarrhea, because they were found in acute phase fecal specimens from the patients. Some of the P/P particles seemed to be new diarrheal agents. The tentatively named Osaka-agent in this study may be a new type of small virus particle. These particles were 35–40 nm (average 40 nm) in diameter with many spikes on their surface, as shown in Fig. 1-F and 2-C. This agent was first detected in October, 1980. Subsequently it was found sporadically in infantile cases and also in cases in outbreaks of acute gastroenteritis in a nursery home and primary schools. We concluded that it was an etiological agent of acute gastroenteritis from the facts that it was detected in fecal specimens collected in the acute phase of diarrhea from patients during these outbreaks, that a specific antibody response against the agent was observed in paired sera of patients by the immune electron microscopic method, and that no bacterial infection was observed in these cases. On PTA-negative staining, this agent appeared morphologically similar to Otofuke agent (Urasawa, 1984) and Tottori agent, as small spherical viruses in Japan were compared (Ishida et al., 1981), and also to minireovirus (Middleton et al., 1977) and minirotavirus (Spratt et al., 1978), both from Canada, and another agent from Glasgow, Scotland (Madeley et al., 1977), although these were smaller. Norwalk-like agent and coronavirus particles were not detected, presumably because these agents had not yet spread to this district.

Of the six viruses shown in Table 1, rotavirus was the commonest, being responsible for about 30% of all cases and 67% of virus-positive cases. In contrast, viruses other than rotavirus were found in only 15% of all cases; adenovirus in 6% and small spherical agents in 9%. Therefore, rotavirus seemed the most important viral agent associated with diarrhea even in this district. In addition, rotavirus might have interfered with infection by astrovirus, calicivirus or adenovirus, although a few cases of infection with these viruses were also found in the winter between January and March. Rotavirus showed marked seasonal variation, as shown in Fig. 3, being detected in cooler seasons, but not in summer each year. This feature may be common in temperate zones of the world, as described by many workers (Kapikian et al., 1976; Birch et al., 1977; Middleton et al., 1977; Hieber et al., 1978; Konno et al., 1978; Brandt et al., 1982; Brandt et al., 1983; Konno et al., 1983).

In contrast to rotavirus, other viruses seemed to show no seasonal variation, although Osaka-agent was found mainly in the cooler months of October to December (Fig. 4). There were some cases of infections with caliciviruses in July, in the middle of the summer. This is consistent with the report of Suzuki et al. (1979) of a few cases of calicivirus infection in June in Yamagata Prefecture of Japan. These results suggest that rotavirus shows a seasonal variation each year, while other viruses do not. These findings must reflect the geographic distributions of these viruses associated with infantile diarrhea. Fig. 5 shows close correlations between infections with rotavirus and other viruses and the temperature and/or relative humidity. Infection with rotavirus may be significantly affected by the weather as reported by others (Middleton et al., 1977; Hieber et al., 1978; Brandt et al., 1982; Moe and Shirley, 1982; Konno et al., 1983).

The age distribution of viral gastroenteritis observed in this study suggests that infants of 0 to 2 years old are very susceptible to infection by rotavirus and other viruses, whereas young children of over two years old are less susceptible, possibly due to aquisition of immunity against these viruses, as evidenced by findings on rotavirus infection (Blacklow et al., 1976; Oishi et al., 1979).

In this survey, 8 children were found to be infected with two different viruses within ten months (Table 4). This finding indicates that there is no antigenic cross reaction between different viruses, for examples between Osaka-agent and calicivirus in Case no. 4, and between astrovirus and rotavirus in Case nos. 2 and 8. But no cases of reinfection with rotavirus were observed in the three year survey period. Rotaviruses collected in this district were classified into two distinct electropherotypes by analysis of their double-stranded RNAs by polyacrylamide gel electrophoresis. The electrophoretic patterns were formerly named short and long patterns (Espejo et al., 1979; Kalica et al., 1981), corresponding to subgroups 1 and 2 of human rotavirus, respectively, distinguished by antigenic analysis (Kutsuzawa et al., 1982; Thouless et al., 1982). Thus it seems possible that infants could be reinfected with an antigenically different strain.

Attempts to cultivate these viruses were successful only with two isolates of adenovirus type 5 (Ad-5). The two patients from which Ad-5 was isolated must also have had respiratory infection, because Ad-5 has usually been isolated from nasopharyngeal specimens, not from fecal ones unless the patients have acute respiratory infection (Fox et al., 1969); some of fastidious adenoviruses detected in this survey may be enteric adenoviruses, as described in recent reports (Gary et al., 1979; Johansson et al., 1980; Yolken et al., 1982; Chiba et al., 1983).

The fastidious viruses including adenoviruses associated with acute gastroenteritis can be detected mainly by EM. Large amounts of virus particles are necessary for studies on their epidemiological and biological characteristics including their antigenic properties and molecular natures. Thus a better method is required for their cultivation as refered to the successful cultivations of some human rotaviruses (Sato et al., 1981; Urasawa et al., 1981; Wyatt et al., 1983), astrovirus (Lee and Kurz, 1981) and calicivirus (Cubitt and Barrett, 1984).

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